

Vitamin A Modulation of Xenobiotic-induced Hepatotoxicity in Rodents

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Vitamin A (VA, retinol) has been shown to modulate cells of the immune system. When rats are pretreated with VA (75 mg/kg/day) for 7 days, there is greatly potentiated liver damage upon subsequent exposure to hepatotoxicants such as CCl_4 . This potentiated damage can be blocked by superoxide dismutase or catalase, suggesting that reactive oxygen species are playing a major role in the increased liver injury. The studies reported here examined VA-induced modulation of CCl_4 hepatotoxicity in different strains of male rats, female rats, and different strains of male mice. Also, the role of VA-induced weight loss on potentiation of CCl_4 injury was investigated. Rats or mice were dosed with VA (retinol) at 75 mg/kg/day, po, for 7 days. In an additional VA dose-response study, mice were given VA at 18.8, 37.5, or 75 mg/kg/day, po, for 7 days. On day 8 they were given a dose of CCl_4 which elicited mild hepatic damage. On day 9 they were necropsied. Male and female Sprague-Dawley rats, and male Fischer-344 and athymic nude rats pretreated with VA had an approximately 10-fold increase in liver damage as compared to vehicle controls. Pretreatment of male Balb/C, C3H/HeJ, Swiss-Webster, or athymic nude mice resulted in a marked reduction of CCl_4 -induced hepatic damage. In the dose-response study in mice, increasing doses of VA elicited increasing amounts of protection from CCl_4 -induced liver injury. Paired feeding studies revealed that VA-induced weight loss (or decreased weight gain) had no effect on subsequent VA-induced potentiation (rats) or protection (mice) from hepatic damage caused by CCl_4 . These results indicate that VA-induced potentiation of CCl_4 hepatotoxicity is similar in male and female Sprague-Dawley rats and in male T-lymphocyte-deficient nude rats. However, in mice, VA pretreatment results in protection from CCl_4 -induced liver injury. The results also show that the VA associated weight loss has no effect on modulation of hepatic injury in rats or mice.—*Environ Health Perspect* 102(Suppl 9):39–43(1994)

Key words: vitamin A, retinol, carbon tetrachloride, rats, mice, Kupffer cells, paired feeding

Introduction

Many chemicals, such as carbon tetrachloride, allyl alcohol, and acetaminophen are known to cause liver damage upon exposure. Likewise, numerous compounds, such as vitamin A (retinol), endotoxin (lipopolysaccharide), and methyl palmitate have been demonstrated to modulate the function of the immune system after their administration (1,2). Little is known, however, about the role of the immune system in the progression of liver damage and even less well characterized are the effects of immune system modulators on xenobiotic-induced hepatotoxicity.

Kupffer cells [(KC), resident macrophages of the liver] and neutrophils polymorphonuclear (PMN) are phagocytic white blood cells that have recently been shown to play a role in liver injury (3–5). Studies suggest that the contribution of KC

or PMN to liver injury varies depending on the hepatotoxic agent. Regardless of which type of white blood cell is involved, release of cytotoxic factors are responsible for increased hepatocellular damage. Examples of cytotoxic factors are reactive species of oxygen (ROS) such as superoxide anion and nitric oxide, and proteases (6,7). Presumably, when hepatocytes or hepatic nonparenchymal cells (NPC) are damaged, chemical mediators are released which stimulate KC and PMN to release ROS and proteases resulting in increased injury to surrounding hepatocytes and NPC. Therefore, immunomodulators could affect KC and PMN in one of two ways; either to suppress their function resulting in decreased hepatic damage or to increase their function, causing increased or potentiated damage. Experiments in this laboratory have been principally focused on vitamin A-induced modulation of hepatotoxicity caused by xenobiotic compounds.

Vitamin A (VA, retinol) is found in many foods and is available as an over-the-counter food supplement. Besides its requirement for normal cellular function, it has been shown to be beneficial as an adjunct to cancer chemotherapy and in the prevention of some types of skin cancer. Among its physiological effects, VA (as retinol or retinoic acid) can activate macrophages *in vivo* and *in vitro* (2,8).

Original investigations in this laboratory sought to determine the consequences

of high doses of VA on the subsequent exposure to a hepatotoxicant such as carbon tetrachloride (CCl_4). It was discovered that the administration of VA (retinol) by oral gavage to male Sprague-Dawley rats at a dose of 75 mg/kg/day for 7 days, greatly potentiated the hepatotoxicity of a subsequent ip dose of CCl_4 as determined by increased plasma ALT activity and increased histologic hepatic damage (9). In a VA dose-response study in rats, increasing doses of VA from 30 to 75 mg/kg/day, po, for 7 days, elicited increasing potentiation of CCl_4 -induced liver injury (10). Administration of superoxide dismutase (SOD) or catalase eliminated the potentiated damage, implicating release of ROS by KC as the cause of the increased damage (9). In addition, when KC isolated from VA-pretreated rats were stimulated *in vitro*, they produced significantly greater amounts of superoxide anion as compared to KC from controls (11). However, it was noted that VA-treated rats had reduced weight gain or lost weight during the treatment period, while the corn oil controls had a gain in body weight of approximately 20% during the treatment period. Since the preceding experiments were only performed in male Sprague-Dawley rats and because of concern about weight loss in VA-treated animals, the following experiments were designed to answer three questions. First, do other strains of rats, and do female rats exhibit the same VA-induced

This article was presented at the IV European ISSX Meeting on Toxicological Evaluation of Chemical Interactions: Relevance of Social, Environmental, and Occupational Factors held 3–6 July 1992 in Bologna, Italy.

The authors thank Charleen Prytula for typing this manuscript and Jeanette O'Hare for graphic arts. This research was supported, in part, by a grant from National Institutes Health (ES06095).

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Table 1. Plasma ALT activities in athymic nude, male and female Sprague-Dawley, and F-344 rats.

Treatment group ^a	Plasma ^a LT Activities (mean ± SE)							
	Male Nude		Male Sprague-Dawley		Female Sprague-Dawley		Male Fischer-344	
Veh/Veh	36 ± 1	(n = 3)	28 ± 2	(n = 2)	31 ± 4	(n = 2)	26 ± 4	(n = 4)
VA/Veh	36 ± 2	(n = 3)	32 ± 4	(n = 2)	46	(n = 1)	17 ± 1	(n = 4)
Veh/CCl ₄	734 ± 79	(n = 3)	221 ± 40	(n = 4)	752 ± 286	(n = 4)	366 ± 58	(n = 4)
VA/CCl ₄	5341 ± 1219*	(n = 3)	2746 ± 293*	(n = 4)	8351* ± 1874	(n = 4)	4024* ± 284	(n = 4)

^aPretreated with VA (75 mg/kg/day, po) for 7 days followed by CCl₄ (0.2 ml/kg or 0.1 ml/kg [F-344], ip). *, Significantly different from Veh/CCl₄ group at *p* < 0.05.

potentiation of CCl₄ hepatotoxicity as male Sprague-Dawley rats? Second, would another species of laboratory animal (i.e., the mouse), have increased hepatotoxicity with VA pretreatment? Third, does the decrease in body weight of VA-treated animals contribute to the increased hepatotoxicity present in CCl₄-treated animals?

Materials and Methods

All animals were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed in polycarbonate cages with hardwood bedding in an AALAC-approved facility and allowed to acclimate for at least 1 week before use. They were given free access to food (Teklad, Harlan Inc., Indianapolis, IN) and tap water and were maintained on a 12-hr light–dark cycle. Rats used in these studies were male Fischer-344 (F-344), male athymic nudes (nu from hooded stock) and male and female Sprague-Dawley. Males weighed 250 to 350 g and females 225 to 275 g before the start of dosing. Mice were male, Swiss-Webster, B6C3F1, Balb/C, C3H/HeJ (endotoxin insensitive), and athymic nudes (nu from albino stock) weighing approximately 25 g.

Aquasol A Drops (retinol, Astra Pharmaceutical Products, Westborough, MA) or its vehicle (10% propylene glycol, 7% Tween 20 in distilled water) or retinol (Fluka Chemical Co., Ronkonkoma, NY) dissolved in corn oil (rats in paired-feeding study) were administered to each animal by oral gavage at a dosage of 75 mg/kg/day. In a VA dose–response study, male Swiss-Webster mice were dosed with vehicle or Aquasol A at 18.8, 37.5, or 75 mg/kg/day,

po. In mice, doses of VA greater than 75 mg/kg/day for 7 days were judged to be too toxic for evaluation. Rats and mice were dosed for 7 consecutive days. On day 8 rats were dosed with corn oil (vehicle) or CCl₄ (Aldrich Chemical Co., Milwaukee, WI) at 0.2 ml/kg (nude and Sprague-Dawley) or 0.1 ml/kg (F-344), ip. Because of the sensitivity of mice to CCl₄-induced liver damage, its very steep-dose response in this species, and due to strain differences, three different doses of CCl₄, administered by the ip route, were necessary to obtain comparable degrees of liver injury in these mouse strains. Therefore, on day 8 Balb/C mice were dosed with CCl₄ at 12.5 µl/kg, Swiss-Webster mice at 20 µl/kg and C3H/HeJ and athymic nude mice at 15 µl/kg. Twenty-four hours after the administration of CCl₄, the animals were killed by CO₂ inhalation and exsanguinated through the posterior vena cava. Heparinized blood samples were immediately collected and plasma separated for determination of ALT activities using a Beckman DU-7 spectrophotometer (Sigma kit No. 59-20, Sigma, St. Louis, MO). A section of liver was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 to 6 µm, stained with hematoxylin and eosin and examined microscopically.

In paired-feeding studies, male Sprague-Dawley rats (250–300 g) or male Swiss-Webster mice (25 g) were individually housed. To ensure that each VA-vehicle animal ate the same amount as a VA-treated, the amount of rodent-chow eaten by each VA-treated animal was weighed daily, and the same percentage of food per

gram body weight was given to its pair-fed VA-vehicle control animal the next day. With regard to VA and CCl₄ dosing, and necropsy procedures, these animals were treated identically to those above.

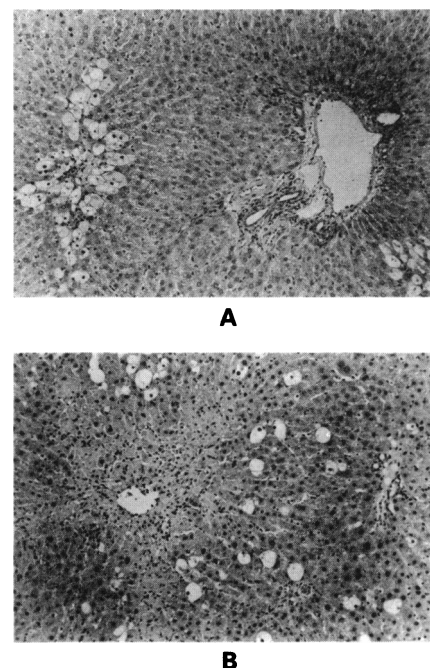


Figure 1. Photomicrographs of livers from male Sprague-Dawley rats pretreated with vitamin A (75 mg/kg/day, po) for 7 days. On day 8, they were treated with CCl₄ (0.2 ml/kg). Necropsies were performed on day 9. H&E × 33. (A) Veh/CCl₄; there is severe vacuolization, enlargement, and necrosis of hepatocytes adjacent to the central vein. (B) VA/CCl₄; there is vacuolization, enlargement and degeneration of midzonal hepatocytes and severe necrosis of centrilobular hepatocytes.

Table 2. Plasma ALT activities in male Balb/C, C3H/HeJ, athymic nude, and Swiss-Webster mice pretreated with Vitamin A followed by CCl₄ treatment.

Group	Plasma ALT activities (mean ± SE)							
	Balb/C		C3H/HeJ		Athymic nude		Swiss-Webster	
Veh/Veh	15 ± 2	(n = 5)	15 ± 1	(n = 3)	17 ± 1	(n = 3)	34 ± 7	(n = 5)
VA/Veh	20 ± 4	(n = 5)	30 ± 3	(n = 3)	24 ± 12	(n = 3)	20 ± 7	(n = 5)
Veh/CCl ₄	7777* ± 2468	(n = 5)	1081 ± 834	(n = 6)	2134* ± 1074	(n = 6)	2177* ± 480	(n = 9)
VA/CCl ₄	1992 ± 1374	(n = 5)	124 ± 65	(n = 6)	52 ± 11	(n = 6)	520 ± 233	(n = 9)

Pretreated with VA (75 mg/kg/day, po) for 7 days followed by CCl₄. Carbon tetrachloride doses: Balb/C = 0.0125 ml/kg, ip; Swiss-Webster = 0.02 ml/kg, ip; C3H/HeJ and athymic nude = 0.015 ml/kg, ip; *significantly different from VA/CCl₄ group at *p* < 0.05.

Statistical evaluation was performed using a one-way analysis of variance (ANOVA) of the Number Cruncher Statistical System (NCSS).

Results

Pretreatment of male F-344, athymic nude, and male and female Sprague-Dawley rats for 7 days with VA (75 mg/kg/day, po) resulted in potentiation of hepatic damage as determined by increased plasma ALT activities and histological examination of liver sections. Although CCl_4 at this dose (0.2 ml/kg ip) caused greater baseline toxicity in male athymic nude and female Sprague-Dawley rats as compared to male Sprague-Dawley and F-344 rats, VA-pretreatment resulted in an approximately 10-fold increase in CCl_4 -induced hepatotoxicity. Histologically, rats treated with vehicle/ CCl_4 had mild centrilobular damage in the two to four rows of hepatocytes surrounding the central vein. These cells were greatly enlarged and contained several large, clear intracytoplasmic vacuoles. Hepatocytes immediately adjacent to the central vein were necrotic with shrunken, fragmented eosinophilic cytoplasm and pyknotic nuclei.

With VA pretreatment, this damage was more extensive with severe degeneration and necrosis that included both centrilobular and midzonal hepatocytes (Figure 1).

Pretreatment of male Swiss-Webster, Balb/C, C3H/HeJ (macrophage impaired), and athymic nude mice for 7 days with VA (75 mg/kg/day, po) resulted in protection from CCl_4 -induced hepatic damage as determined by plasma ALT activities and microscopic examination of liver sections. Balb/C and Swiss-Webster mice had an approximately 4-fold protection, while C3H/HeJ had approximately 10-fold protection, and athymic nude mice, a 40-fold protection (Table 2). The VA dose-response study in male Swiss-Webster mice revealed that as the daily dose of VA increased, from 18.8 to 75 mg/kg/day, the degree of protection against CCl_4 -induced liver injury also increased (Figure 2). Histologically, mice treated with vehicle/ CCl_4 had moderate degeneration and necrosis of centrilobular hepatocytes extending to midzonal regions, similar to that seen in rats. With VA pretreatment, the centrilobular hepatic damage was much reduced with minimal hepatocyte damage

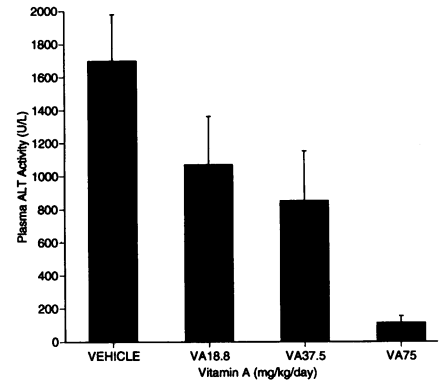
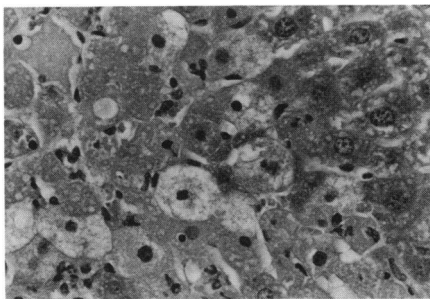


Figure 2. Plasma alanine aminotransferase (ALT) activities in male Swiss-Webster mice. The mice were treated by gavage with vehicle or VA at 18.8, 37.5, or 75 mg/kg/day, po, for 7 days. On day 8, they were administered CCl_4 at 20 $\mu\text{l/kg}$, ip. Plasma was analyzed for ALT activity 24 hr following the dose of CCl_4 . $n = 4$ for each VA/ CCl_4 group; $n = 14$ for vehicle/ CCl_4 group.

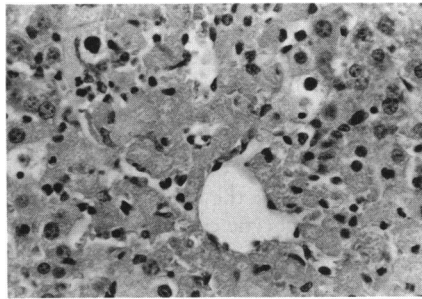
around the central vein in Swiss-Webster and Balb/C mice and virtually no hepatocyte damage seen in C3H/HeJ or athymic nude mice (Figure 3).

In the paired feeding study, rats fed *ad libitum* and dosed with VA-vehicle gained



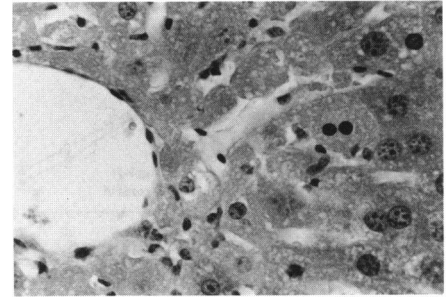
A

(A) Swiss-Webster, Veh/ CCl_4 0.02 ml/kg. There is severe degeneration and necrosis of centrilobular hepatocytes.



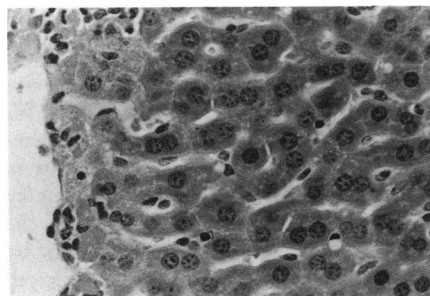
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(C) C3H/HeJ, Veh/ CCl_4 0.015 ml/kg. There is severe degeneration and necrosis of centrilobular hepatocytes with infiltration of numerous leucocytes.



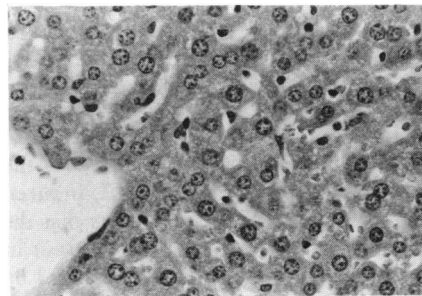
E

(E) Athymic Nude, Veh/ CCl_4 0.015 ml/kg. Centrilobular hepatocytes are degenerate with numerous intracytoplasmic vacuoles and nuclear pyknosis.



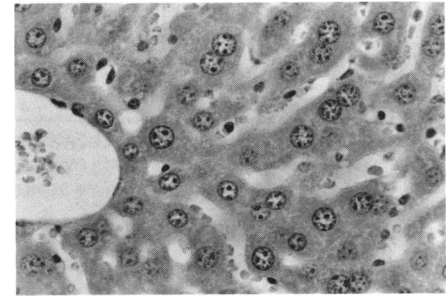
B

(B) Swiss-Webster, VA/ CCl_4 0.02 ml/kg. Only a few hepatocytes immediately adjacent to the central vein are vacuolated and degenerate.



D

(D) C3H/HeJ, VA/ CCl_4 0.015 ml/kg. Centrilobular hepatocytes appear normal.



F

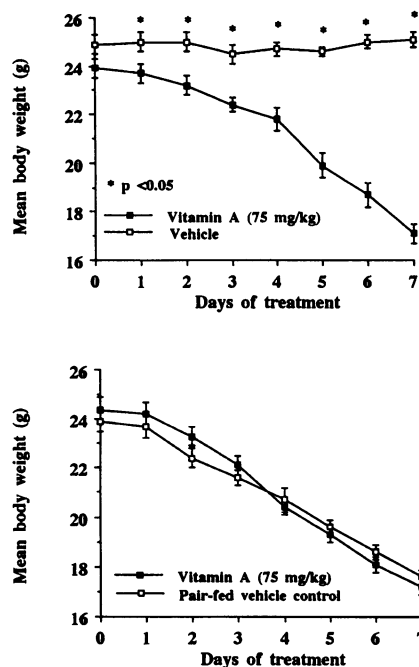
(F) Athymic Nude, VA/ CCl_4 0.015 mg/kg. Centrilobular hepatocytes appear normal.

Figure 3. Photomicrographs of liver from male mice pretreated with vitamin A (75 mg/kg/day), po, for 7 days. On day 8 they were treated with CCl_4 . Necropsies were performed on day 9. H&E $\times 132$

Table 3. Plasma ALT activities in male Sprague-Dawley rats pretreated with vitamin A or its vehicle combined with paired-feeding to one group of vehicle-treated animals.

Treatment group	Weight gain, g	Plasma ALT activities, U/L
Veh/CCl ₄	40 ± 4	327 ± 23
Veh/CCl ₄ (pair-fed)	17 ± 4	346 ± 26
VA/CCl ₄	6 ± 2	3214* ± 422

VA (75 mg/kg/day po) for 7 days or its vehicle followed by CCl₄ (0.2 ml/kg, ip). Mean ± SEM. *, significantly different from Veh/CCl₄ and Veh/CCl₄ (pair-fed) groups at $p < 0.05$.

**Figure 4.** Mean body weight of mice during 7 days of oral gavage with VA (75 mg/kg/d) (■) or its vehicle (5 ml/kg/day) (□). The upper graph demonstrates the typical weight loss due to VA treatment. The bottom graph demonstrates that pair-fed controls lost an equal amount of body weight. Each symbol is the mean ± SEM of 10 animals.**Table 4.** Plasma ALT activities in male Swiss-Webster mice pretreated with vitamin A or its vehicle combined with paired-feeding to one group of vehicle-treated animals.

Treatment group	Plasma ALT activities, U/L
Veh/CCl ₄	2236 ± 303
Veh/CCl ₄ (Pair-fed)	1413 ± 748
VA/CCl ₄	583* ± 144

VA (75 mg/kg/day, po) for 7 days or its vehicle followed by CCl₄ (0.2 ml/kg, ip). Mean ± SEM. *, significantly different from Veh/CCl₄ and Veh/CCl₄ (pair-fed) groups at $p < 0.05$.

40 ± 4 g during the course of the study. Animals given daily doses of VA gained only 6 ± 2 g during the study, while rats pair-fed to the VA-treated rats only gained 17 ± 4 g during treatment. Following dosing with CCl₄ (0.2 ml/kg ip), VA-vehicle and pair-fed rats had minimal elevations in plasma ALT activities (approximately 325–350 U/L) while VA-pretreated rats had an approximate 10-fold increase of plasma ALT activities (Table 3). Male Swiss-Webster mice and their pair-fed vehicle controls both lost approximately 25% of their body weight over the 7 day VA dosing period (Figure 4). However, when dosed with CCl₄, VA-pretreated mice had an approximately 10-fold reduction in plasma ALT activities as compared to both the pair-fed and non-pair-fed CCl₄ groups (Table 4).

Discussion

Vitamin A (retinol) is a widely used, over-the-counter food supplement. Since it is fat soluble and stored in the Ito cells of the liver, many adults have increasing hepatic stores of it through their lifetimes (12). Studies over the last decade have shown that VA can modulate elements of the immune system, including macrophages. Vitamin A and other retinoids (retinal and retinoic acid) stimulate peritoneal macrophages to increased phagocytosis and interleukin production (2) and alveolar macrophages to increased phagocytosis and tumoricidal activity (8). Retinol, retinoic acid, and retinyl palmitate have been shown to increase the release of superoxide anion by phagocytic cells (13,14). In turn, the secretory products of stimulated peripheral blood monocytes and macrophages, including KC, have been shown to be involved in hepatic injury following exposure to hepatotoxins (6,7, 15). Superoxide dismutase (SOD) and catalase (CAT) degrade superoxide anion and hydrogen peroxide, respectively. Methyl palmitate (MP) administration inhibits the function of macrophages, including Kupffer cells. In this laboratory, it has been demonstrated that the administration of SOD, CAT, or MP to rats alleviates the VA-potentiated increase in CCl₄-induced hepatic damage (9). This suggests that the potentiated liver damage is due, at least in part, to reactive oxygen species released by these phagocytic cells.

In the studies reported here, VA potentiated CCl₄-induced hepatotoxicity to a similar degree in male and female Sprague-Dawley rats and in F-344 and athymic nude rats. Vitamin A potentiation of CCl₄ in athymic nude rats suggests that potentia-

tion can occur in the absence of T-lymphocytes in this strain. Fischer-344 rats have been shown to be more susceptible to pulmonary oxidant-induced injury (16). Such a strain difference may also be expressed in the liver of F-344 rats since Smith et al. (17) have shown them to be more susceptible to the hepatotoxic effects of the redox cycling compound, diquat. In our VA model, they responded in a similar manner as both the Sprague-Dawley and athymic nude rats, except that they required a lower dose of CCl₄ to produce a comparable degree of liver injury.

Mice responded differently than rats in this particular model of chemical induced hepatotoxicity. As seen in the control animals in these studies, mice are more susceptible to CCl₄-induced hepatotoxicity than rats. Doses required to produce comparable elevations in serum ALT activities were approximately one-tenth of those required for rats. However, the most surprising result was that pretreatment of mice with VA protected mice from CCl₄-induced liver injury.

Mice exhibited considerable strain variability with respect to VA protection. Balb/C and Swiss-Webster mice had an approximately 4-fold protection; C3H/HeJ (macrophage impaired), 10-fold protection; and athymic nude mice (T-lymphocyte deficient), 40-fold protection. In the dose-response study in Swiss-Webster mice, increasing doses of VA up to the highest possible dose elicited increasing protection from CCl₄-induced liver injury. This is in contrast to rats, which show increasing potentiation of CCl₄ with increasing doses of VA (10). The mechanism(s) responsible for VA-associated protection of CCl₄-induced hepatotoxicity in mice remain(s) to be elucidated. It is possible that differences in retinol metabolism and tissue distribution between rats and mice may explain, in part, the species differences reported here. In rats, hepatic retinol and retinyl palmitate concentrations increased following VA treatment (10). We are currently determining hepatic concentrations of retinol and retinyl palmitate in mice following VA treatment at 75 mg/kg for 7 days. In addition, studies of the effects of VA on CCl₄ metabolism by murine hepatocytes and on phagocytic cell function are currently in progress.

It was observed that oral gavage with VA resulted in weight loss in mice and a reduction of weight gain in rats. Since VA can cause anorexia and weight loss in humans, this finding was not unexpected (18). Therefore, paired feeding studies

were conducted in both species to determine the role of food intake in VA modulation of CCl₄-induced hepatotoxicity. In both rats and mice, vehicle control animals, pair-fed to VA animals, had reductions in weight gain (rats) or decreases in body weight (mice) similar to the VA animals. However, when dosed with CCl₄, there was no alteration of liver injury. Therefore, in these studies, VA-associated weight loss or decrease of weight gain was not associated with VA modulation of CCl₄-induced liver injury. Recent studies in our laboratory indicate that VA administration to rats may induce increased production of tumor

necrosis factor- α (TNF- α , data not shown). Since TNF- α (19) can result in weight loss, this is a possible cause of the weight loss or decreased weight gain (rats) that was observed.

These studies demonstrate that large doses of VA can modulate the hepatotoxic effects of subsequent administration of CCl₄. The wide variability of responses between species and among strains of the same species (mice) make it difficult to predict the responses that will be seen in other species, including humans. However, retinoids have been reported to increase macrophage and monocyte activity in peo-

ple. After consumption of excessive amounts of VA some humans could be exposed to other immunomodulators and also to potentially toxic chemicals. Therefore, it will be important to better characterize how VA and other immunomodulators interact with the wide variety of chemicals (drugs, environmental pollutants, etc.) to which people are exposed and to determine the mechanisms that explain the differences observed in mice and rats with respect to the effects of VA on chemical induced hepatotoxicity. Such information will assist in the extrapolation of these results obtained in animals to humans.

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